PCT/GB00/01211

GB00/1211

4

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

09/937714

REC'D 19 MAY 2000
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Signed Andrew Gregg

Dated 13 APR 2000

Patents Form 1/77

Patents Act ... 7
(Rule 16)

THE PATENT OFFICE
31 MAR 1999
RECEIVED BY HAND

The Patent Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference CORCORAN/J/99/001/ABH — 31 MAR 1999

2. Patent application number *(The Patent Office will fill in this part)* **9907461.9**

01APR99 E457000-1 C50659

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

KING'S COLLEGE LONDON,
AN INSTITUTION INCORPORATED
BY ROYAL CHARTER, OF
STRAND, LONDON WC2R 2LS.

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

5947499004

4. Title of the invention

NEURITE REGENERATION

5. Name of your agent *(if you have one)*

KCL ENTERPRISES LTD,
WATERLOO BRIDGE HOUSE,
57 WATERLOO ROAD,
LONDON SE1 8WA.

D Young & Co
21 New Fetter Lane
London
EC4A 1DA
51177 6-400
eau

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

06985354003

Patents ADP number *(if you know it)*

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
	—	

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*
—	

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

NO

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	23
Claim(s)	—
Abstract	—
Drawing(s)	10

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 30/3/99

12. Name and daytime telephone number of person to contact in the United Kingdom DR ADAM HAJJAR 0171-872-3465

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*

b) *Write your answers in capital letters using black ink or you may type them.*

c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

e) *Once you have filled in the form you must remember to sign and date it.*

f) *For details of the fee and ways to pay please contact the Patent Office.*

NEURITE REGENERATION

This invention relates to the regeneration of neurites and more particularly to the stimulation of neurite outgrowth in the peripheral and central nervous systems.

INTRODUCTION

Retinoids are a family of molecules derived from vitamin A and include the biologically active metabolite, retinoic acid. The cellular effects of RA are mediated through the action of two classes of receptors, the retinoic acid receptors (RARs) which are activated both by all-*trans*-RA (*t*RA) and 9-*cis*-RA (9-*cis*-RA), and the retinoid X receptors (RXRs), which are activated only by 9-*cis*-RA (Kastner et al., 1994;Kleiwer et al., 1994). The receptors are of three major subtypes, α, β and γ, of which there are multiple isoforms due to alternative splicing and differential promoter usage (Leid et al.). The RARs mediate gene expression by forming heterodimers with the RXRs, whilst the RXRs can mediate gene expression as homodimers or by forming heterodimers with a variety of orphan receptors (Mangelsdorf & Evans, 1995). Many studies on a variety of embryonic neuronal types have shown that RA can stimulate both neurite number and length (review, Maden, 1998). as, indeed, can the neurotrophins (Campenot, 1977; Lindsay, 1988; Tuttle and Mathew, 1995). The neurotrophins are a family of growth factors that are required for the survival of a variety of neurons of primary sensory neurons in the developing peripheral nervous system (Snider, 1994). One of the earliest genes induced by NGF in PC12 cells is the orphan receptor NGFI-B

(NURR1) (Millbrandt, 1989). This suggests that the growth factor and retinoid mediated pathway in developing neurons can interact.

We have used embryonic mouse DRG to investigate further the nature of the interaction between the retinoid mediated pathway and the growth factor pathway to determine which of the RARs and RXRs are expressed in neurons that are dependent upon different neurotrophins for their survival. We have shown that RARβ2 is up-regulated in neurons that respond to RA by increasing neurite outgrowth and by the use of receptor selective agonists only the RARβ agonist will substitute for RA in inducing neurite outgrowth. These results therefore indicate a rolefor RA acting via RARβ2 in the outgrowth of neurites from certain classes of neurons.

In the procedures described throughout this specification the materials used are either described in the literature, or available commercially, or available on request from the Developmental Biology Research Centre, The Randall Institute, Kings College, 26-29 Drury Lane, London WC2B 5RL, UK. (M.Maden or J.Corcoran). The primers used in pcr procedures are described in the Appendix given at the end of this specification.

MATERIALS & METHODS

DRG cultures : DRG were obtained from E13.5 mice, freed of non-ganglionic tissue and collected in ice-cold calcium magnesium free phoshate buffered saline. To prepare dissociated cell suspensions the ganglia were treated with 0.05 % trypsin for 15 minutes at 15 °C. The reaction was stopped by the addition of 1% serum and single cells obtained by trituration with a 23 G needle. The cells were then spun at 1000 g for ten minutes and resupended in media. They were plated out at

a density of approximately of 25000 cells/cm^2 in wells that had been precoated first with 100µg/ml poly D lysine for 2 hrs and secondly with 100µg/ml laminin for 30 minutes. The cultures were fed every 2 days. Culture media consisted of DMEM-F12 with glutamine (Gibco), 6 % glucose, ITS (Gibco). The growth factors used were either 50ng/ml NGF (7s, Promega) 50ng/ml NT3(promega) or 50ng/ml BDNF. Retinoids were used at a concentration of 1×10^{-7} M. CD366 activates RARα, CD2019 activates RARβ, CD437 activates RARγ and CD2809 activates all of the RXRs.

RT-PCR analysis : RNA was extracted (trizol, Gibco) and cDNA prepared by the use of a pharmacia kit as described in the manufacturers instructions. The primers used were from mouse RARs RXRs and GAPDH (details upon request). PCR was carried out for 30 cycles. Amplification was carried out as follows, denaturation for 30 s at 95 °C, annealing for 30 s at 55°C and extension for 1 min at 72°C. One fifth of the resultant product was then run on a gel and blotted. This was then probed with the appropriate RAR and RXR and GAPDH for normalisation.

Human treatments

From the experiments we describe below we conclude that the upregulation and activation of RARβ2 is essential for neurite outgrowth.

RESULTS

Response to RA

Neurons were grown in serum free medium for a period of two days before adding 1×10^{-7}M RA to the cultures. The cultures were then fixed

three days later and analysed for neurite out growth with the monoclonal antibody NF200. In the absence of RA the BDNF neurons (Fig. 1 E) grew neurites whilst the NGF and NT-3 neurons (Fig. 1 A, C) showed little or no neurite outgrowth. In contrast when RA was added to the medium there was a increase in the length and number of neurites in the NGF and NT-3 neurons (Fig. 1 B, D) whilst RA did not effect the BDNF neurons (Fig. 1 F).

Expression of receptors and response to RA by RT-PCR

There was no difference in the expression of the RXRs in neurons cultured either in serum free medium or serum free plus RA (data not shown). In contrast there were variations in the RAR receptor profiles. Each of the three types of neurons expressed RARα 1 (Fig. 2 A, B, C, lane 1) and this receptor was up-regulatied strongly in response to RA in the NGF and NT-3 neurons and slightly up-regulated in the BDNF neurons (Fig. 2 A, B, C, lane 8). Of the four possible RARβ isoforms only the RARβ2 isoform was detected in each of the three types of neurons (Fig. 3 A, B, C, lane 2) and this was strongly upregulated by RA in the NGF and NT-3 neurons (Fig. 3 A, B, lane 6) but not in the BDNF neurons (Fig. 3 C, lane 6). The only RARγ isoform detected in the neuronal cultures was RARγ1. This was strongly expressed in the NGF and NT-3 neurons (Fig. 4 A, B, lane 1) and was found to be down regulated in response to RA (Fig. 4 A, B, lane 8). No RARγ isoform was detected in the BDNF neurons either in the absence or presence of RA (data not shown).

Receptor selective analogues and neurite outgrowth

This data demonstrated that upon RA addition BDNF neurons were unaffected whereas NGF and NT-3 neurons up-regulated RARβ2 and

down-regulated RARγ1. This suggested that the up-regulation and activation of RARβ2 is responsible for the increase of neurite outgrowth that we saw in the NGF and NT-3 neurons (Fig. 1 B, D). In order to test this hypothesis we used receptor selective syntheticretinoids which have been developed specifically to activate individual receptors. CD366 activates RARα, CD2019 activates RARβ, CD437 activates RARγ and CD2809 activates all of the RXRs.

The RARβ agonist increased neurite outgrowth in the NGF and NT-3 neurons (Fig. 5 B, F), but did not effect neurite outgrowth in the BDNF neurons (Fig. 5 J). None of the other agonists increased neurite outgrowth in any of the neuronal populations (Fig. 5 A, C, D E, G, H, I, K, L).

FIGURE LEGENDS

Figure 1. Effect of RA on neurite outgrowth from DRG neurons. DRG neurons were cultured either in the presence of NGF, NT-3 or BDNF for a period of two days at which point 1 x 10^{-7} M RA was added. They were then stained for neurite outgrowth after five days with NF200 antibody. A. NGF B NGF + 1 X 1O-7 M RA. C. NT-3, D. NT-3 + 1 X $1O^{-7}$ E. BDNF F. BDNF + 1 X $1O^{-7}$ M RA

Figure 2. Expression of RARα isoforms in DRG neurons cultured either in the absence or presence of RA. DRG neurons were cultured in the presence of either NGF, NT-3 or BDNF for a period of two days, 1 X 10-7 M RA was then added and the presence of the RARa isoforms were then assayed by RT-PCR. A. NGF neurons B. NGF neurons + 1 X 10^{-7} M RA C NT-3 neurons D. NT-3 neurons + 1 X 10^{-7} M RA E. BDNF neurons F. BDNF neurons + 1 X 10^{-7} M RA. Lanes: 1 RARα1 2 RARα2 3 RARα3 4.RARα4 5. RARα5 6. RARα6 7. RARα7

Figure 3. Expression of RARβ isoforms in DRG neurons cultured either in the absence or presence of RA. DRG neurons were cultured in the presence of either NGF, NT-3 or BDNF for a period of two days, 1 X 10-7 M RA was then added and the presence of the RARb isoforms were then assayed by RT-PCR. A. NGF neurons B. NGF neurons + 1 X 10^{-7} M RA C NT-3 neurons D. NT-3 neurons + 1 X 10^{-7} M RA E. BDNF neurons F. BDNF neurons + 1 X 10^{-7} M RA. Lanes: 1 RARβ1 2 RARβ2 3 RARβ3 4. RARβ4

Figure 4. Expression of RARγ isoforms in DRG neurons cultured either in the absence or presence of RA. DRG neurons were cultured in the presence of either NGF, NT-3 or BDNF for a period of two days, 1 X 10-7 M RA was then added and the presence of the RARg isoforms were then assayed by RT-PCR. A. NGF neurons B. NGF neurons + 1 X 10-7 M RA C NT-3 neurons D. NT-3 neurons + 1 X 10-7 M RA E. BDNF neurons F. BDNF neurons + 1 X 10-7 M RA. Lanes: RARγ1 2 RARγ2 3 RARγ3 4. RARγ4 5. RARγ5 6. RARγ6 7. RARγ7

Figure 5. Effect of RAR and RXR agonists on neurite outgrowth from DRG neurons. DRG neurons were cultured either in the presence ofNGF , NT-3 or BDNF for a period of two days at which point either 1 x 10-7M RA of a RARα, RARβ, RARγ or RXR agonist were added to the cultures. Cultures were then stained for neurite outgrowth at five days with the NF200 antibody. A- D NGF type neurons E-H NT-3 type neurons I-J BDNF type neurons, agonists RARα A, E I, RARβ, B, F, J., RARγ C, G, K, RXR D, H, L.

Nerve growth factor (NGF) stimulates neurite outgrowth from cultured adult dorsal root ganglia (DRG). The vitamin A derivative all-*trans*-retinoic acid (tRA) induces neurite outgrowth from various embryonic sources, including DRG. Here we show that NGF is upstream of tRA. When adult mouse DRG are cultured in the presence of NGF and an inhibitor of tRA synthesis, neurite outgrowth does not occur. Conversely, when tRA is added along with a blocking antibody to NGF, neurite outgrowth occurs as normal. We further show that NGF induces transcription of both the tRA-synthesizing enzyme RALDH-2 and the retinoic acid receptor-β2 as well as detectable release of synthesized tRA. We deduce that tRA is required for adult DRG neurite regeneration and that NGF acts upstream of RA to induce its synthesis.

METHODS

DRG cultures: whole DRG were obtained from adult mice of approximately six months old. These were cultured in cellogen matrix (ICN flow), prepared by mixing 1 volume of 7.5 % sodium bicarbonate, 1 volume of 10x MEM (Gibco) and 8 parts cellogen (ICN flow). The pH was adjusted to 7.5 by dropwise addition of 5M NaoH. The cultures were fed every 2 days. Culture media consisted of DMEM-F12 with glutamine (Gibco), 6 % glucose, GMS-A (Gibco) and 10% delipidated serum. The supplements used were 100ng/ml NGF (7s, Promega), 1 x 10^{-7} M RA (sigma), 1 x 10^{-5} M disulphiram, 1/500 dilution of blocking antibody against NGF (chemicon); and dimethyl sulphoxide. Cultures were fed every two days, on the fifth day they were fixed in 4 % paraformaldyde and stained with the neurofilament antibody, NF200 (sigma). In parallel

experiments the DRG were either collected for PCR analysis or they were sonicated before been placed on the F9 reporter cells.

RT-PCR analysis: RNA was extracted (trizol, Gibco) and cDNA prepared by the use of a pharmacia kit as described in the manufactures instructions. The primers used were from mouse RALDH-2, mouse GAPDH and mouse RARB (details upon request). PCR was carried out for 30 cycles for RALDH-2 and 25 cycles for RARβ2. Amplification was carried out as follows, denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 1 min at 72°C. One fifth of the resultant product was then run on a gel and blotted. This was then probed with cDNAs coding either for RALDH-2 or RARβ2 and GAPDH for normalisation.

Reporter cell assay: the generation and characterisation of the F9 reporter cells has been described [21] For the assay the cells were grown on gelatin coated wells of a 24-well culture dish (Nuclon) to confluence. Just prior to introducing the sonicated DRG to the wells the growth medium was aspirated from the F9 cells and replaced with 0.5ml serum-free medium. After overnight incubation the cultures were fixed and the β-galactosidase expression was visualised. They were gently washed twice with room temperature PBS, then fixed for 5 minutes at 4°C with 2% paraformaldehyde, 0.2% glutaraldehyde in PBS. They were rinsed gently twice more then covered with 1ml of X-gal stain per dish and incubated at 37°C overnight. The X-gal stain was replaced with PBS and the blue stained cells counted on an Olympus inverted microscope fitted with an eyepiece graticule. The cells were counted in three random fields for each of the three experiments.

Human treatment

Retinoids that are RARβ2 agonists can be applied topically over the damaged area or given orally. A suitable dose is in the range 0.025 %-0.1% in creams or given orally at 1-2 mg/kg body weight.

RESULTS

Adult mouse DRG were cultured in the presence of NGF (100ng per ml), or tRA (100 nM) for five days. In both cases neurite outgrowth occurred (Fig. 6b and data not shown). Little or no neurite outgrowth occurred in adult DRG cultured in only delipidated serum (Fig. 6a). Differences in number of neurites were significant (Fig. 7a; 1, 2 and 3). When tRA was added together with NGF, there was no additive effect of the two treatments (Fig. 6c), and no significant difference was seen between tRA, NGF or tRA plus NGF groups (Fig. 7a; 2, 3 and 4).

To show that NGF induces RA we cultured adult DRG in the presence of NGF and 10 μM disulphiram, a compound which blocks the conversion of retinaldehyde to tRA by inhibiting the enzyme retinaldehyde dehydrogenase. As shown in Fig. 6d, addition of disulphiram completely abolished NGF-induced neurite outgrowth compared to NGF alone (Fig. 7a, 2 and 5) or NGF plus DMSO (vehicle for disulphiram) (Fig. 7a, 2 and 6). To confirm that disulphiram did not affect cell survival within the explants, we performed two types of rescue. Explants were cultured for eight days in medium supplemented with disulphiram. In the first rescue, tRA was added to the explants from the beginning of the experiment; in the second, tRA was added on day 4. Neurite outgrowth occurred in both of the rescued cultures and not in the disulphiram alone cultures (Figs. 6e, f and 7b).

Inhibition of the inductive effect of NGF but not of tRA by disulphiram suggests that NGF may precede tRA in the cascade leading to neurite outgrowth. To test this, we used a blocking antibody against NGF. In the presence of NGF and the blocking antibody, virtually no neurite outgrowth occurred (Fig. 6g; compare to DRG cultured in the presence of NGF alone, Fig. 6b). On the other hand, DRG cultured in the presence of the NGF-blocking antibody and tRA (Fig. 6h) showed neurite outgrowth equivalent to that obtained with NGF alone (Figs. 6b and 7c).

If NGF is upstream of RA, it should induce synthesis of tRA after addition to DRG cultures. To test this we used an F9 reporter cell line that responds to the presence of tRA due to transfection with 1.8 kb of the mouse RARβ2 gene promoter containing a retinoic acid response element linked to the *lacZ* gene (Maden, M., Sonneveld, E., van der Saag, P. T. & Gale, E. *Development* 125, 4133–4144 (1998). In the presence of tRA, activated cells can be detected after β-galactosidase histochemical staining. NGF itself does not activate these cells because in the presence of NGF there was no labeling of the F9 cells above background. We then cultured adult DRG in delipidated serum for five days under three different conditions: no NGF, with NGF or NGF plus the NGF-blocking antibody. The DRG were then sonicated and placed on the F9 reporter cells. NGF-treated DRG homogenates produced a clear RA signal relative to untreated DRG (Fig. 7d). This activation was prevented when the DRG were cultured with blocking antibody in addition to NGF (Fig. 7d).

We next showed which tRA synthesizing enzyme might be induced by NGF. Retinol is converted by a two-step oxidative process firstly to retinaldehyde and then to retinoic acid. It has been shown that retinaldehyde dehydrogenase type 2 (RALDH-2) is expressed in the developing nervous system. Using RT-PCR, we found strong induction of

RALDH-2 by NGF in cultured adult DRG (Fig. 7e). Lastly, we also found up-regulation of the RARβ2 receptor in NGF-stimulated cultures (Fig. 7e) which we have shown to be required for neurite outgrowth.

Our results show that tRA can stimulate neurite outgrowth from an adult neural tissue, the DRG. NGF similarly stimulates neurite outgrowth from this tissue, and we have demonstrated that it does so by inducing tRA synthesis via an enzyme, RALDH-2. The sequence of events in the induction of neurite outgrowth by NGF is: NGF ----› RALDH-2 ----› tRA ----› RARβ2 ----› neurite outgrowth.

Neurotrophins have been considered as potential agents for induction of nerve regeneration and treatment of neurodegenerative diseases, but a major problem for their use is lack of effective modes of delivery to the site of the injury. Because tRA is required for the regenerative response and it is downstream of NGF, then the problem of delivery could be overcome as tRA is a low-molecular-weight lipophilic compound that can be administered orally. Thus, tRA is proposed for clinical use in neurology.

FIGURE LEGENDS

Figure 6. Neurite outgrowth in adult mouse DRG cultured for five (a–d, g, h) or eight days (e, f) in the presence of delipidated serum plus: (a) no addition; (b) NGF, 100 ng per ml; (c) NGF and 100 nM tRA; (d) NGF and 10 mM disulphiram; (e) disulphiram and tRA added on day 0; (f) disulphiram; (g) NGF and blocking antibody (h) NGF-blocking antibody and tRA.

Figure 7. Neurite numbers (a-c), tRA synthesis (d) and gene induction (e) in adult mouse DRG after various treatments. (a) Effects of NGF, tRA and disulphiram at five days (1, no additive; 2, NGF, 100 ng per ml; 3, tRA, 100 nM; 4, NGF, 100 ng per ml and tRA, 100 nM; 5, 100 ng/ml NGF and 10 mM disulphiram; 6, NGF, 100 ng per ml and DMSO). Error bars = s.e.; $n = 6$. $*p < 0.01$; $**p < 0.0001$; Student's t-test. (b) tRA rescue of DRG treated with 10 M disulphiram (1, no tRA; 2, 100 nM tRA on day 0; 3, 100 nM tRA on day 4). $n = 6$. (c) NGF-blocking antibody on 5-day DRG cultures. 1, NGF, 100 ng per ml; 2, NGF plus blocking antibody; 3, blocking antibody plus 100 nM tRA. $n = 4$. (d) Increase in percentage β-gal-positive F9 cells in response to cultured DRG. 1, no additive; 2, NGF, 100 ng per ml; 3, NGF plus blocking antibody. $n = 9$. (e) RT-PCR analysis of RALDH-2 enzyme and RARβ2 expression in adult DRG cultured with (lane 2) or without (lane 1) NGF (100ng per ml) for five days. GAPDH was used to indicate presence of cDNA in both samples.

We have demonstrated above that the retinoic acid receptor β2 is a crucial transducer of the retinoic acid signal to stimulate the outgrowth of neurites in embryonic and adult dorsal root ganglia. We have also discovered that in embryonic mouse spinal cord the same situation exists, RARβ2 is up-regulated at concentrations which maximally stimulate neurite outgrowth. In the adult mouse spinal cord, however, no such up-regulation is observed and no neurites are extended *in vitro*. But when the adult cord is transfected with RARβ2 then neurite outgrowth occurs. It does not occur when the cord is transfected with another isoform of RARβ, RARβ4 thus showing a high level of specificity of receptor function.

The failure of CNS axons to regenerate under normal circumstances has been attributed to one or a combination of causes: the low abundance of neurotrophic factors; the absence of growth-promoting molecules; the presence of growth-inhibiting molecules. However, in the following description we show that by manipulating the genome of the neurons themselves, a stimulation of neurite outgrowth can be obtained from adult mouse spinal cord *in vitro*. We have transfected the adult spinal cord with a nuclear transcription factor, the retinoic acid receptor-β2 (GenBank ref. nos. x56573, MMRARB2), and as a result transformed the normally inert spinal cord into one which can extend neurites. Therefore, the manipulation of both the neuronal genome and the environment may lead to more complete spinal cord regeneration than either manipulation alone.

METHODS

Cultures. Spinal cord was disected from either E13.5 or 10 month old mice and cut into transverse pieces of about 5 mm. These were cultured in cellogen matrix (ICN flow), prepared by mixing 1 volume of 7.5 % sodium bicarbonate, 1 volume of 10x MEM (Gibco) and 8 partscellogen (ICN flow). The pH was adjusted to 7.5 by dropwise addition of 5M NaoH. Explants were fed every two days. The media consisted of DMEM-F12 with glutamine (Gibco), 6 % glucose, GMS-A (Gibco) 10% delipidated serum and all-*trans*-RA (stock solution, 1 x 10^{-5} M, Sigma). On the fifth day they were fixed in 4 % paraformaldyde and stained with the neurofilament antibody, NF200 (Sigma).

RT-PCR analysis. RNA was extracted (trizol, Gibco) and cDNA prepared by the use of a Pharmacia kit as described in the manufactures instructions. The primers used were from GAPDH, RARβ2 and RARβ4. PCR was carried out for 30 cycles for embryonic spinal cord and 40 cycles for adult spinal cord. Amplification was carried out as follows, denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 1 min at 72°C. One fifth of the resultant product was then run on a gel.

Transfections. Virus stocks were prepared and *B* galactosidase staining carried out as described by Lim, F., Hartley, D., Starr, P., Song, S., Lang, P., Yu, L., Wang, Y.M. & Geller, A.I. Use of defective herpes-derived plasmid vectors. *Meth.Mol. Biol.* 62, 223-232 (1997).

The titres used were: pHSV RARβ2, 5 x 10^{-4} ip/ul, pHSVRARβ4,, 4 x 10^{-4} ip/ ul, pHSVlacz 5 x 10^{-4} ip/ul .

Human Treatment

The method of treatment involves insertion of the human RARβ2 cDNA into the defective herpes simplex viral vector. This is then placed at the site of injury by injecting the construct into both ends of the injured cord. The dose used is in the range of 4×10^{-4} - 5×10^{-4} ip/ul. The duration of the treatment will depend upon the severity of the injury.

RESULTS

Effect of RA on embryonic mouse spinal cord in vitro.

E13.5 spinal cord was dissected from mouse embryos placed in acellogen matrix and cultured in 10 % delipidated serum. All-*trans*-RA was added at 3 different concentrations (10^{-8}M, 10^{-7}M, 10^{-6}M) and after 5 days the explants were stained with a neurofilament antibody and examined for the presence of neurites. There was an increasing number of neurites emerging from the cultured cord with increasing concentrations of RA with the maximal effect at 10^{-6}M (Fig. 8C, E, G). To demonstrate that the induction of neurites involved the up-regulation of RARβ2, RT-PCR was performed on cultures after 5 days in the same range of RA treatments. This revealed that RARβ2 is normally expressed in embryonic spinal cord at all concentrations of RA used (Fig. 9A, lanes 1-5) and that it is strongly up-regulated after 1×10^{-6} M RA treatment (Fig. 9A, lane 5), the same concentration which gives maximal neurite outgrowth.

Lack of effect of RA on adult mouse spinal cord in vitro.

An identical series of experiments was performed using 10 month old adult spinal cord rather than the embryonic cord. In contrast to the embryonic cord, RA had no effect on neurite outgrowth at any concentration tested and like the untreated controls, these RA treated

adult cords failed to extend any neurites at all (Fig. 8B, D, F, H). Examining the involvement of RARβ2 by RT-PCR revealed that control adult spinal cord had little or no detectable endogenous levels of this receptor (Fig. 9B, lane 1) and that there was no change in its level in response to RA treatment at any concentration (Fig. 9, lanes 2-5), unlike the embryonic cord.

Induction of neurites in adult spinal cord

We have therefore concluded that it was the lack of RARβ2 expression which may be responsible for the completely inert behaviour of the adult spinal cord. To test this further we used a defective herpes simplex virus type 1 (HSV-1) vector to transfect pieces of adult (10 months) mouse spinal cord. Three different transfections were performed, two of which served as controls. Firstly, just the vector containing lacZ (pHSVlacZ); secondly the vector containing RARβ2 (pHSVRARβ2) ; thirdly the vector containing another isoform of the RARb gene, RARβ4 (pHSVRARβ4). The latter served as a very precise control for transfection since we have not detected the RARβ4 isoform after RA treatment of neurons in our previous experiments hence it is not involved in neurite outgrowth. We first ensured that the transfections were successful and that the relevant receptor isoform was expressed in the cultured cord. Pieces of spinal cord were transfected overnight with the appropriate construct and analysed either three or four days later. The pHSVlacZ treated cords showed a significant amount of transfection had taken place as judged by b-galactosidase staining of the adult cord (Fig. 10B). RT-PCR demonstrated that transfection with the RARβ2 vector resulted in the expression of RARβ2 (Fig. 11, lane 3) but not RARβ4 (Fig. 11, lane 4) and transfection with the RARβ4 vector resulted in the expression of RARβ4 (Fig. 11, lane 8) but not RARβ2 (Fig. 11, lane 7). In the non

transfected cord neither RARβ2 or RARβ4 were detected (Fig. 11, lanes 2 and 6).

The effects of these transfections on neurite outgrowth were clear-cut. Transfection with the pHSVlacZ failed to change the behaviour of the cultured adult cord which remained completely un-responsive in terms of neurite outgrowth (Fig. 12A, 12/12 transfections). Similarly, the transfections with pHSVRARβ4 produced no response in the cultured cord which remained inert (Fig. 12C, 12/12 transfections). However, transfections with the pHSVRARβ2 isoform clearly produced a different behaviour and many neurites appeared in the cultures (Fig. 12B, 8/12 transfections). The number of neurites produced varied between 3 and 23. In the pHSVlacZ transfections there was considerable variability in the number of lacZ-positive cells per explant. This suggests that the variability in neurite number may be due to variability in number of cells transfected.

FIGURE LEGENDS

Figure 8. Comparison of the effect of retinoic acid on neurite outgrowth on cultured E13.5 (A, C, E, G) and 10 month old adult spinal cord (B, D, F, H). Pieces of spinal cord were cultured in cellogen in the presence of 10 % delipidated serum and RA for a period of five days. The medium was changed every two days. A, B, no RA; C, D, 1 x 10^{-8} M RA; E, F, 1 x 10^{-7} M RA; G, H, 1 x 10^{-6} M RA.

Figure 9. Expression of RARβ2 in E13.5 and 10 month old adult spinal cord. Pieces of spinal cord were cultured in the presence of various concentrations of RA for a period of five days after which time RT-PCR analysis of RARβ2 was performed. A. E13.5 (lanes 2-5) and B. 10 month old adult spinal cord (lanes 2-5). Lanes: 1. bluescript/HPA II size markers, 2. no RA, 3. 1 x 10^{-8} M RA, 4. 1 x 10^{-7} M RA, 5. 1 x 10^{-6} M RA. The presence of GAPDH was used to indicate equal amounts of cDNA in the samples.

Figure 10. Transfection of adult spinal cord with pHSVlacZ. Cultured 10 month old adult spinal cord was transfected with 5 x 10^{-4} ipu/ul pHSVlacZ overnight and analysed for *B* galactosidase staining 3 days later. A. non-transfected adult spinal cord. B. adult spinal transfected with pHSVlacZ.

Figure 11. Transfection of adult spinal cord with either pHSVRARβ2 or pHSVRARβ4. Adult spinal cord was cultured in cellogen and transfected either with 5 x 10^{-4} ipu/ul of pHSVRARβ2 or 4 x 10^{-4} ipu/ul pHSVRARβ4 overnight. RT-PCR analysis four days after transfection, of RARβ2 (lanes 2-4) and RARβ4 (lanes 6-8) expression in adult spinal cord

transfected with Lanes 2, 6 no virus , 3, 7 pHSVRARβ2, 4, 8, pHSVRARβ4. The presence of GAPDH was used to indicate equal amounts of cDNA in the samples. Lanes 1,2 bluescript/HPA II size markers.

Figure 12. Effect of either pHSVlacZ, pHSVRARβ2 or pHSVRARβ4 transfection in cultured adult spinal cord onneurite outgrowth. Ten month old spinal cord was cultured in cellogen and transfected with either 5 x 10^{-4} ipu/ul, pHSVlacZ, 5 x 10^{-4} ipu/ul, pHSVRARβ2 or 4 x 10^{-4} ipu/ul pHSVRARβ4 overnight, and analysed for neurite staining with NF200 4 days after transfection. Cultured spinal cord transfected with A. pHSVlacZ, B. pHSVRARβ2, C. pHSVRARβ4.

CONCLUSION

These results strongly indicate that the RARβ2isoform plays a key role in the induction of neurite outgrowth in response to RA and that this may be a crucial component which fails to be up-regulated in the injured adult CNS. Our conclusion is based upon several experiments involving either regenerating or non-regenerating neuronal tissues and their response to RA. Thus the embryonic mouse spinal cord, the embryonic mouse DRG and the adult mouse DRG all respond to RA by up-regulating RARβ2 and extending neurites. In contrast, the adult mouse spinal cord fails to up-regulate RARβ2 and fails to extend neurites. Furthermore, NGF stimulates neurite outgrowth and acts by up-regulating RARβ2 andneurite outgrowth from embryonic mouse DRG can be stimulated by a RARβ agonist.

These results reveal that when the genome of the neuron itself is manipulated then regeneration can be reawakened. These data support a role of RARβ2 in the regeneration of neurites in the adult CNS and feasibility of gene therapy with this transcript in combination with other treatments to achieve functional recovery of the injured spinal cord.

The invention therefore comprises a method of treatment of neurodegenerative disease in which expression of the retinoic acid receptor RARβ2 is ensured in affected cells or tissues. This may be achieved by treatment with an agonist of the RARβ2 receptor or by gene therapy i.e. insertion of the nucleic acid coding for this receptor into the required site in vivo. The latter may be achieved by incorporation of the receptor cDNA in a suitable vector as in, for example, pHSVRARβ2.

The invention may also be seen as the use of these agents in medication for the treatment of peripheral nervous injuries and spinal cord regeneration e.g. in cases of paraplegia.

APPENDIX

RARalpha reverse primer: 535 tgtagctctctgagcactc 517

RARalpha1 forward strand primer 648 tacgccttcttctttcccc 666

RARalpha2 forward strand primer 376 cttttataaccagaaccgggc 396

RARalpha3 forward strand primer 111 caagtagaagccaggaaagtc 131

RARalpha4 forward strand primer 3 ctaagaagacccacacttctg 23

RARalpha5 forward strand primer 30 aagtgaggtgaaaactggg 48

RARalpha6 forward strand primer 42 ttcacagcctggcataac 59

RARalpha6 forward strand primer 24 gagaaggaagtgagccatc 42

RARbeta reverse strand primer 508 tctctgtgcattcctgctttg 488

RARbeta 1 forward strand primer 180 tggacacatgactcactacc 199

RARbeta 2 forward strand primer 598 atgttctgtcagtgagtccc 617

RARbeta 3 forward strand primer 457 gcatgtcagaggacaactg 475

RARbeta 4 forward strand primer 21 agcctggaaaatgccatc 38

RARGamma reverse strand primer 481 ttacagcttccttggacatgcc 460

RARGamma1 forward strand primer 119 agatgctgagccctagcttc 138

RARGamma2 forward strand primer 73 ttactacgcagagccactgg 92

RARGamma3 forward strand primer 169 ggaagatggaagagggaac 187

RARGamma4 forward strand primer 230 caaatttactgggggttgg 248

RARGamma5 forward strand primer 18 ggctggattttggattgaag 37

RARGamma6 forward strand primer 329 ttctgtcctctcactaccttgg 350

RARGamma7 forward strand primer 85 cattaccgcgagtcactaac 104

GAPDH

forward primer 37 cgtagacaaaatggtgaagg 56

reverse primer 333 gactccacgacatactcagc 314

RALDHII

forward primer 1190 gcttcttcattgacccac 1208

reverse primer 1539 cttcaccgtcaggtctttac 1519

RXRalpha

forward primer 745 gcaaggaccggaatgagaac 764

reverse primer 994 tctaggggcagctcagaaaag 974

RXRbeta

forward primer 1910 agaataaaggggtagtgaagg 1930

reverse primer 2176 catcaatgtccccacttg 2159

RXRgamma

forward primer 713 tgccagtagtagccacgaag 732

reverse primer 966 tgagcagttcattccaccc 948

Figure 1.

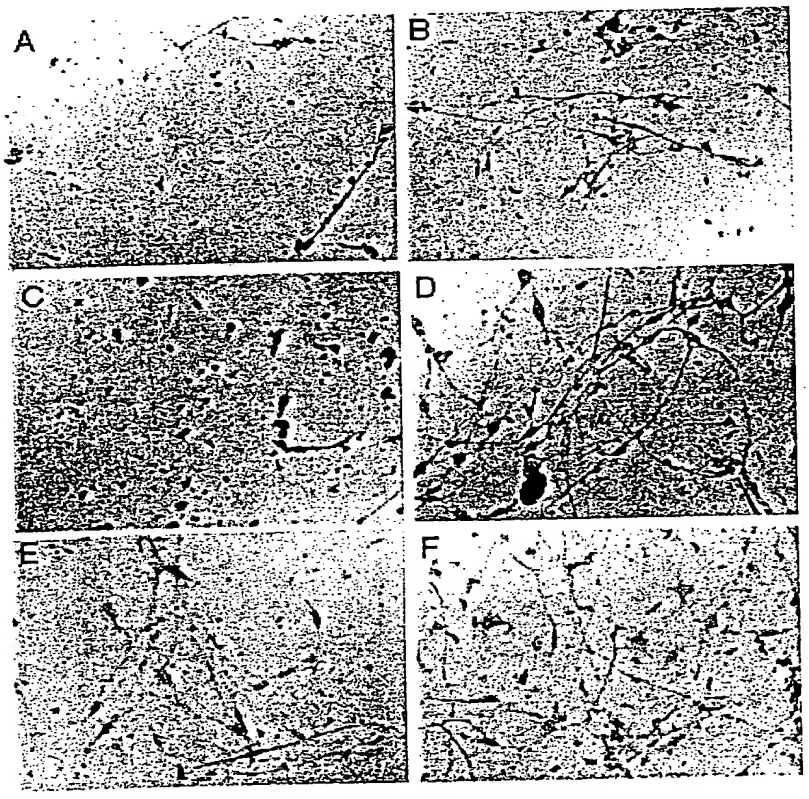


Figure 2.

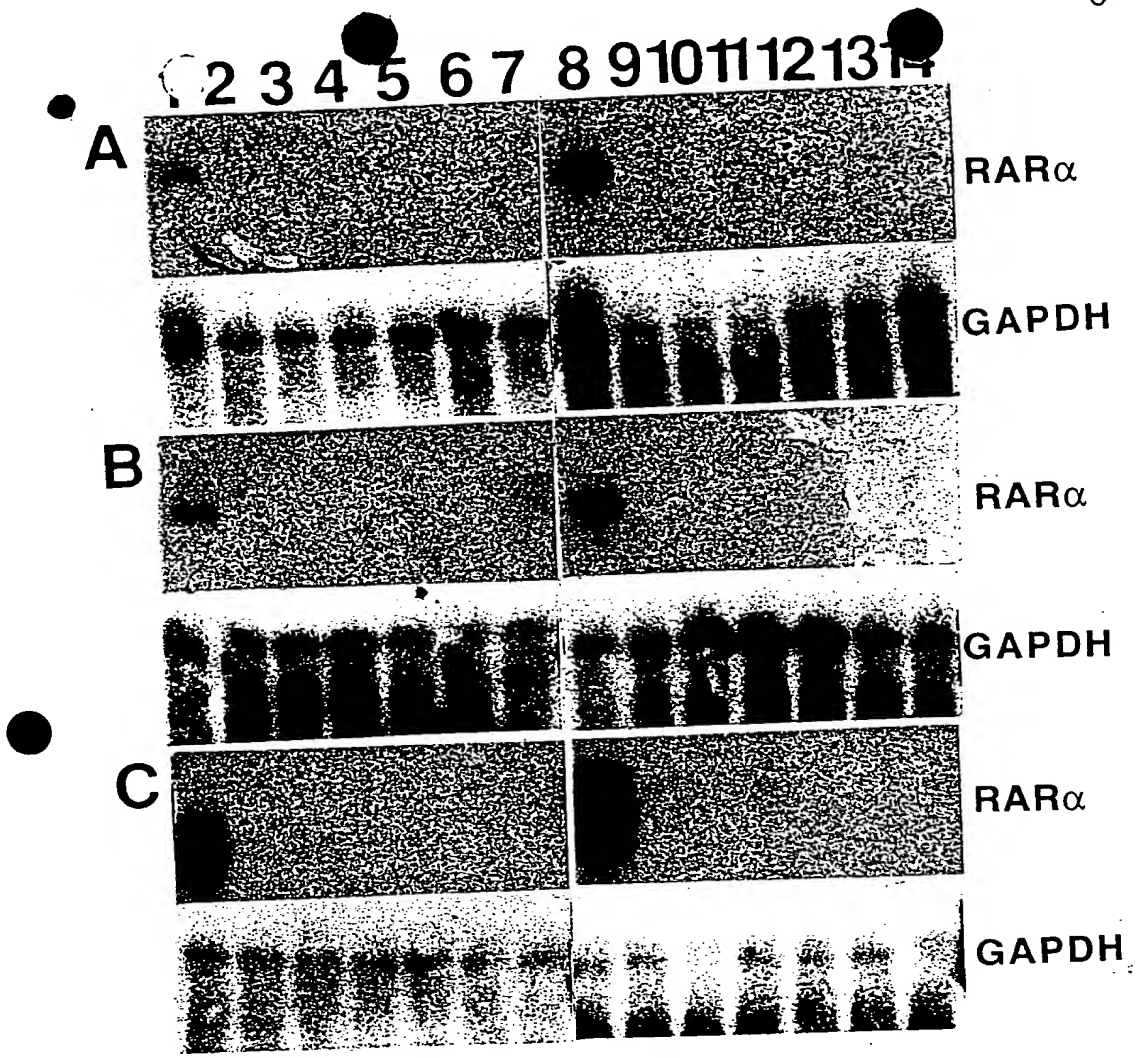
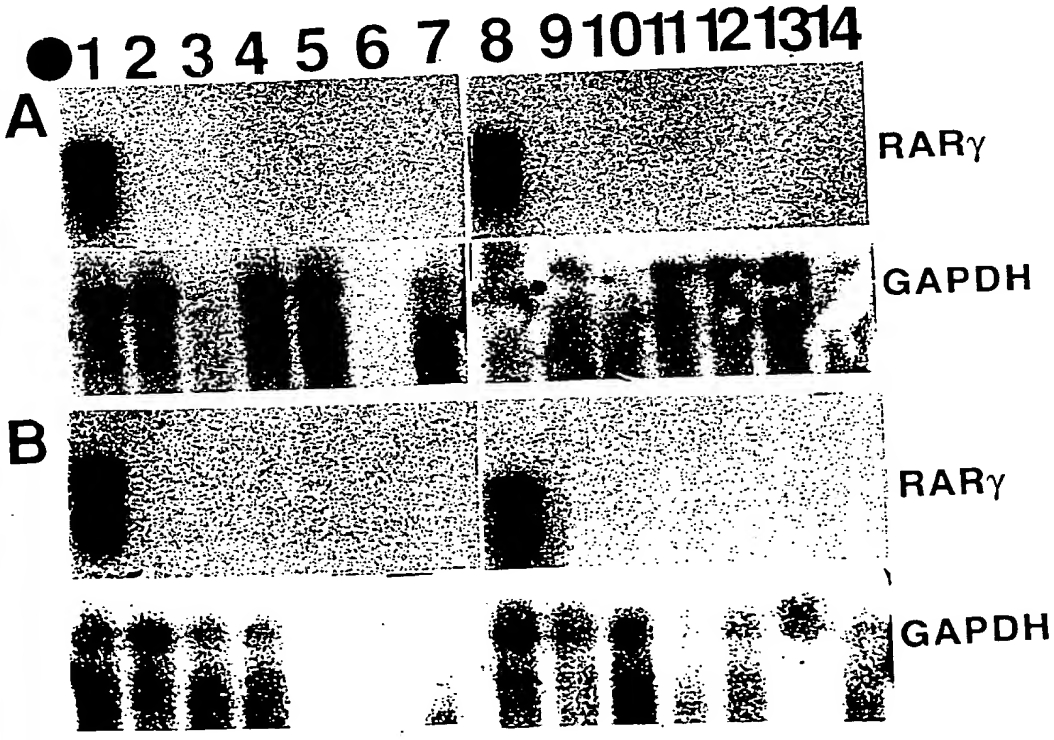
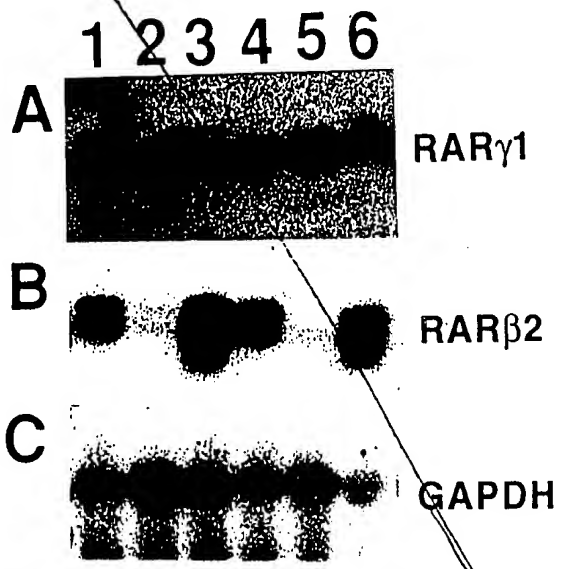


Figure 4



Figures.

Fig 7.



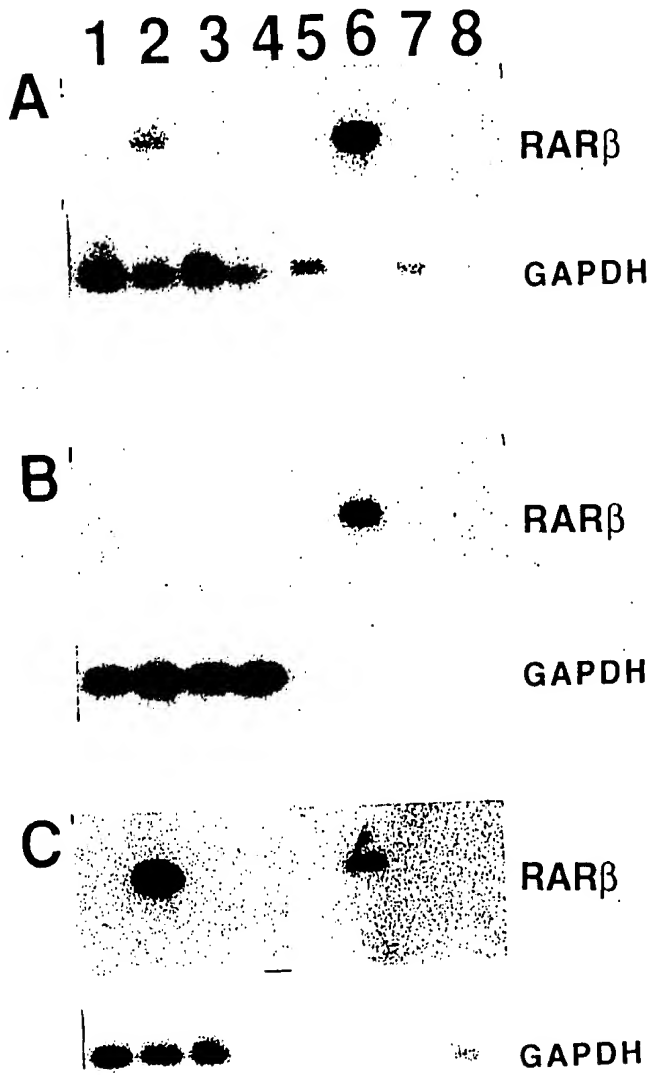
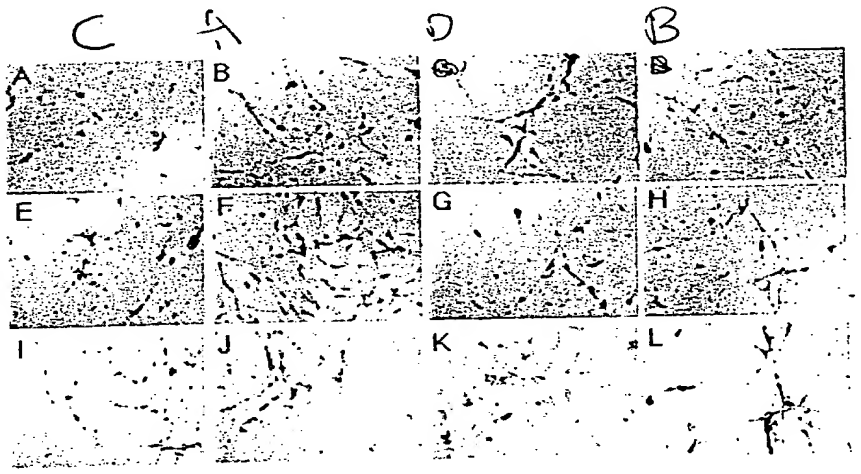


Figure 5.



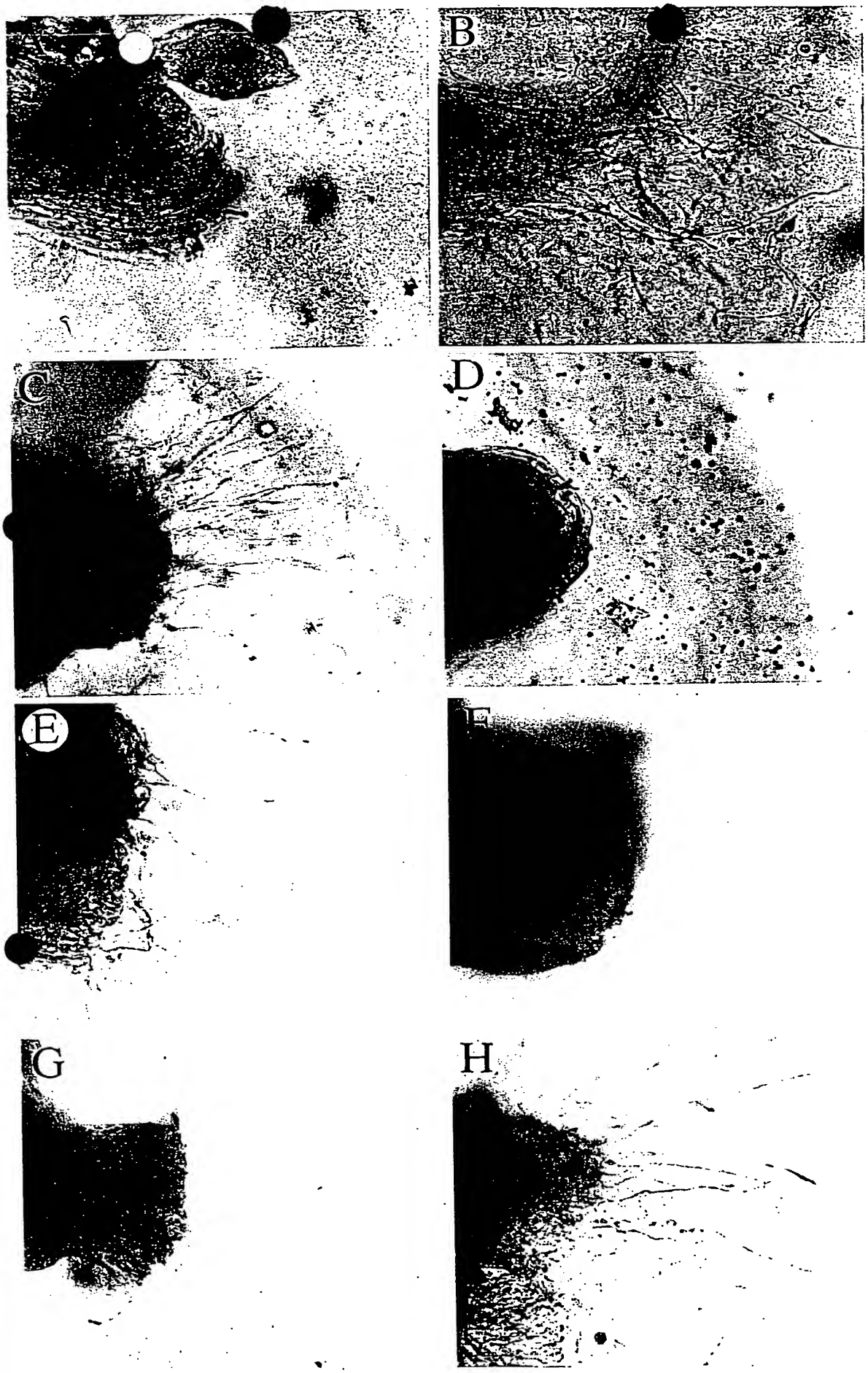
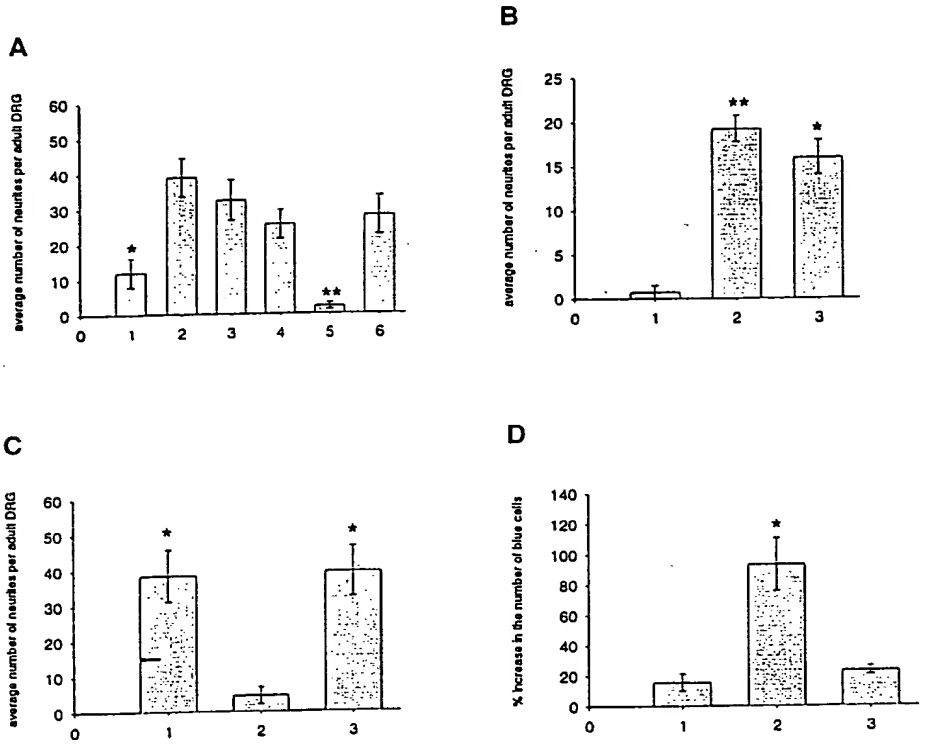
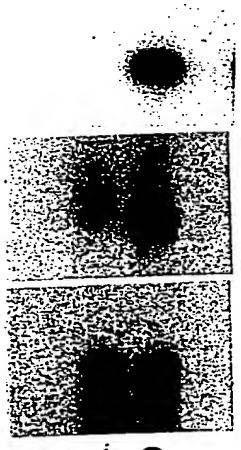


Figure 6.


A
average number of neurites per adult DRG
B
average number of neurites per adult DRG
C
average number of neurites per adult DRG
D
% increase in the number of blue cells

E


RALDH-2
RAR-β
GAPDH

12

Figure 7.

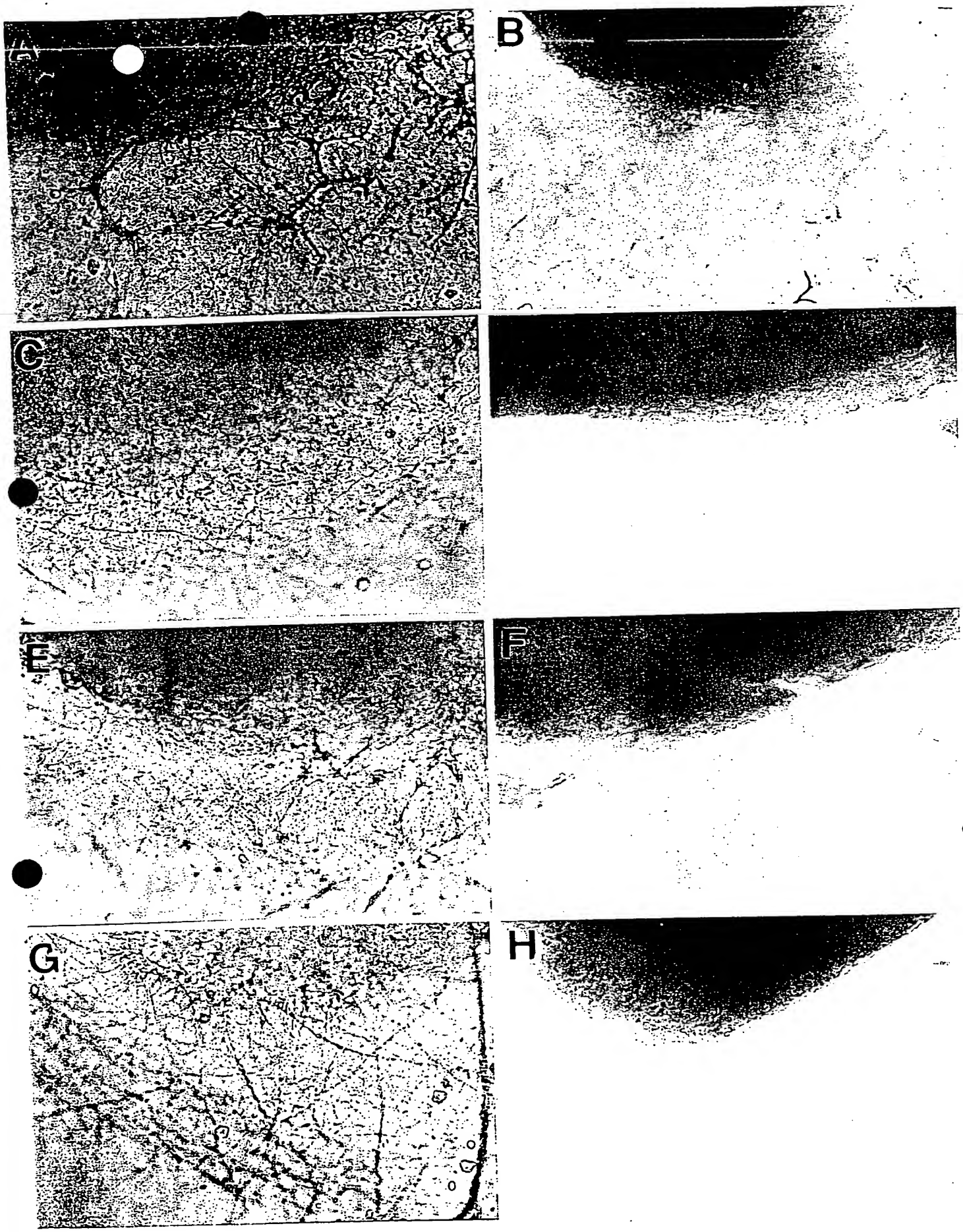


Figure 1.

Figure 11.

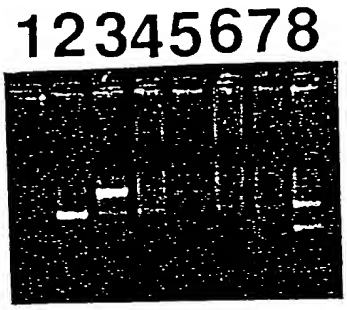
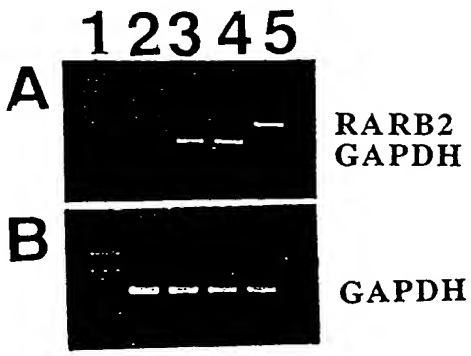


Figure 9.



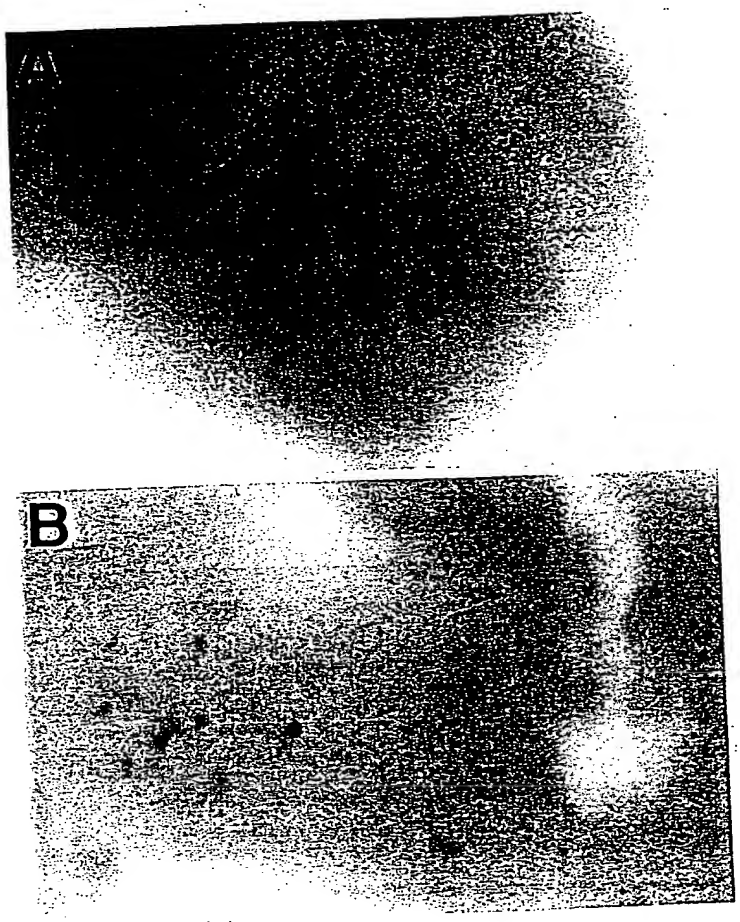


Figure 10.

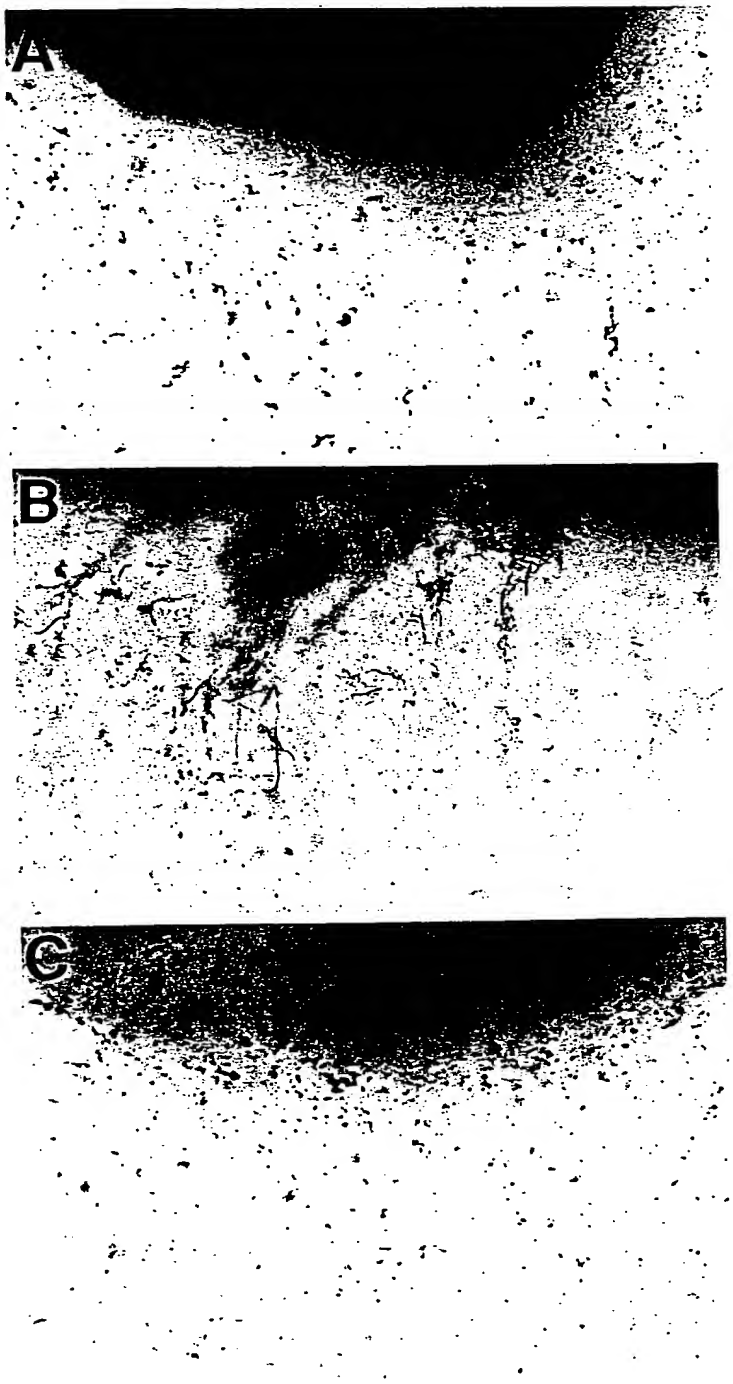


Figure 12.